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Original Research Paper

Low temperature active pectinases production by *Saccharomyces cerevisiae* isolate and their characterization

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ABSTRACT

The objective of the present study was to isolate a yeast strain from fruit wastes and able to produce pectinases by submerged fermentation. Among 5 positive pectinolytic yeast isolates screened, one exhibited strong pectinolytic activity with highest zone (1.6 cm) of pectin hydrolysis in ruthenium red plate assay. This yeast was identified as *Saccharomyces cerevisiae* by morphological characteristics and 26 S rRNA gene analysis. The yeast isolate has been characterised as low temperature active pectinase producer in flasks containing YEPD medium and commercial citrus pectin as substrate. Time course studies of submerged fermentation revealed that the enzymes production is growth associated and maximum enzyme activities were obtained after 48 h of incubation. The strain was able to produce both polygalacturonase (PG) and pectin lyase (PL). The physicochemical properties of pectinases were determined by the study of the effect of temperature and pH on its production and found to be optimum for PG and PL at pH 4.5 and 6.0, respectively, optimum temperature was 30 °C for both the enzymes. The partially purified and characterised enzymes showed the presence of isoforms. Further the enzyme tested for depolymerisation capacity of fruit peel pectin and juice clarification potential. These novel pectinases were low temperature active and moderately thermostable have prospects of application in fruit peel pectin degradation which was studied by using scanning electron microscopy (SEM). Finally we have studied the effect of temperature on clarification of fruit juices, which infers the potential of this yeast-pectinases for commercial and industrial exploitation.

1. Introduction

Pectinases are depolymerising enzymes that degrade pectins present in cell walls of plant tissues (Ismail, 1996). They have widespread applications in the food and textile industries (Henriksson et al., 1999), plant tissue maceration, wastewater treatment and degumming of natural fibres (Baracat-Pereira et al., 1994). In the food industry for clarification of fruit juices, wines (Whitaker, 1984; Alkorta et al., 1998) coffee and tea fermentations (Whitaker, 1984) and extraction of essential oils (Jayani et al., 2005). Microbial pectinases are divided into depolymerising and saponifying enzymes. Depolymerising enzymes are polymethylgalacturonases, pectin lyases, polygalacturonases and pectate lyases. Saponifying enzymes are mainly pectinesterases (Whitaker, 1990). The commercial pectinases used in food industry often come from moulds, in particular the species of the genus *Aspergillus* (*A. niger, A. wentii, and A. oryzae*) and *Rhizopus* (Acuna-Arguelles et al., 1995). Mould pectic enzymes normally contain a mixture of pectinolytic enzymes and are always associated with xylanases, cellulases and hemicellulases which are useful for extraction of juice and maceration of vegetables. However, for the stabilisation of the cloud in orange juice only one type of pectinolytic enzyme is required like high levels of polygalacturonase (Kotzekidou, 1991).

Pectinases have been reported from various yeast species of *Saccharomyces, Kluyveromyces, Cryptococcus, Rhodotorola, Aureobasidium pullulans* and *Candida* (Luh and Phaf 1951; Vaugn et al., 1969; Winborne and Richard, 1978; Lim et al.,1980; Federici, 1985; Barnby et al., 1990; Blanco et al., 1994; Moyo et al., 2003; Da Silva et al., 2005; Merin et al., 2011), especially polygalacturonases (Blanco et al., 1994; Birgisson et al., 2003).Yeasts present an alternative source for the large-scale production of commercial enzymes (Biely and Slavikova, 1994; Blanco et al., 1994; Braga et al., 1998; Jia and Wheals, 2000). They were proved to be advantageous over moulds with regard to the production of pectinases, because of their unicellular nature, simple growth and non-requirement of inducer in growth medium. In addition, gene cloning and gene manipulation may enhance enzyme production, thus suggesting that the commercial enzyme production by yeasts can be promising (Jia and Wheals, 2000). In relation to the

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production of pectinase, yeasts usually do not secret pectin methyl esterase (PME) and, therefore, their pectinases can be used to clarify fruit juice and wine without releasing methanol (Fernandez-Gonzalez et al., 2004; Schwan and Wheals, 2004). Pectinases produced by yeasts are not a mixture as early reported in bacteria and fungi (Pedrolli et al., 2008; Maldonado and Strasser de Saad, 1998), and they have shorter fermentation cycles, hence they are considered as GRAS organisms and are more preferable as production strains. Now-a-days low temperature active enzymes are attractive for usage in fruit juice industry, as colder conditions hamper spoilage. Further milder conditions avoid changes in organoleptic and nutritional properties (Nakagawa et al., 2005). Low temperature active pectinases are helpful in fruit juice clarification (Gainvors et al., 1994) and thermostable enzymes are useful in fruit juice extraction.

Yeasts have a great potential for the production of microbial enzymes for the food industry and they offer an alternative source of bacteria and fungi. The objective of the present study was to select and identify the pectinase-producing yeasts from fruit wastes and to characterise some of the properties of the pectinases produced by the selected yeast.

2. Materials and methods

2.1. Sample collection

Pectinolytic yeasts were isolated from various sources, with special attention given to rotting fruits and fruit wastes obtained from fruit waste dumping yards of fruit juice industries in and around Tirupati. Samples were collected in sterile polythene bags and stored at 4 °C for subsequent studies.

2.2. Screening for pectinolytic yeasts

This was based on the utilisation of pectin as a sole carbon source in the yeast extract peptone dextrose (YEPD) agar, wherein the chief carbon source is replaced by commercial pectin (Sigma). Samples were serially diluted in sterile distilled water before plating onto the medium. The plates were incubated at 25 °C for 72 h. Plates with yeast colonies were isolated and screened for pectinolytic activity using ruthenium red plate assay (Hou et al., 1999). Colonies were flooded with 0.5 mL of 0.02% ruthenium red solution, incubated for one hour at room temperature and washed with sterile water to remove unbound ruthenium. Positive pectinolytic isolates were detected based on clear zones of pectin hydrolysis around the colonies and compared with positive control strain *Saccharomyces bayanus* UCD595.

2.3. Identification of yeast isolates

The pectinolytic yeast isolates were primarily identified by cultural and morphological characteristics by growing on Sabouraud's dextrose agar and Hichrome Candida differential agar and microscopic observation. Colony morphology was observed after 48 h of growth and sugar fermentation characteristics were tested using sugars like glucose, galactose, sucrose, maltose, lactose and melibiose (Campbell, 1973; Kurtzman and Fell, 1998). Molecular identification is carried out for the isolate showing highest polygalacturonase (>1 cm) activity during screening experiments. Total DNA was extracted according to procedures described by Cocolin et al. (2000) and amplified using primers NL1 (50-CATATCAATAAGCGGAGGAAAAG-30) and NL4 (50–GGTCCGT GTTTCAAGACGG-30). Sequencing of the D1/D2 loop region of the 26 S rDNA was performed directly from purified PCR products by the Sequencing Service from BioServe Biotechnologies Pvt Ltd. (Hyderabad, India). The resultant sequences were aligned using the BLAST program for identification (Altschul et al., 1990). The phylogenetic tree was constructed using MEGA 4.0 software. The 26 S rRNA gene partial sequences were submitted to GenBank database with an accession number.

2.4. Maintenance of cultures

Selected yeast isolates were maintained at 4 °C on agar slants containing (g/L): yeast extract (3.0), peptone (5.0), malt extract (3.0) and agar (20) (Schawan and Rose, 1994).

2.5. Production of enzyme

The selected yeast isolate was grown in 500 mL Erlenmeyer flasks containing 100 mL YEPD broth, wherein dextrose was replaced with 1% commercial citrus pectin and inoculated with yeast cells (1×10^4 cells/mL). All media components were of analytical grade and received from Hi media, India. Enzyme production was carried out in submerged fermentation (SmF) by incubating the flasks at 28 ± 2 °C for 72 h on a rotary shaker with a speed of 150 rpm. For the time course studies of enzyme production, samples were collected at every 6 h intervals and assayed for the enzyme activity, protein concentration (mg/mL), biomass and pH variations.

2.6. Effect of temperature and pH on enzyme production

Influence of temperature on enzyme production by *Saccharomyces cerevisiae* isolate 1 was studied by incubating the inoculated flasks at various temperature conditions such as 4, 20, 25, 30, 35, 40 and 45 °C for 48 h. The impact of initial pH of the medium on enzyme production during SmF was studied by adjusting the pH of the culture broth with either 1.0 N HCl or 1.0 N NaOH in a range of 3–8.

2.7. Protein and biomass quantification

Protein quantification was carried out by the modified method of Lowry et al. (1951) using bovine serum albumin (BSA) (Sigma) as a standard. Biomass determination of yeasts was determined gravimetrically after drying cells at 80 °C for 6 h.

2.8. Enzyme assays

Polygalacturonase (PG) activity was assayed by incubating a mixture of 1 mL of 1% polygalacturonic acid (dissolved in 0.05 M acetate buffer, pH 5.0), 8.5 mL of sodium acetate buffer and 0.5 mL of culture filtrate at 45 °C for 1 h in a shaker incubator (Baracat et al., 1989). PG activity was measured by quantifying the amount of reducing sugar groups which had been liberated after incubation with 1% polygalacturonic acid at 45 °C, by the method of DNS (Miller, 1959), using galacturonic acid as a standard. One unit of PG activity (U) was defined as the amount of enzyme that liberates 1 μM of galacturonic acid per min.

Pectin lyase (PL) activity was determined spectrophotometrically by monitoring the increase in absorbance at 240 nm (Albersheim, 1966). The reaction mixture for PL activity contains 1 mL of 0.5% pectin (Hi media, India) dissolved in 0.1 M citrate phosphate buffer of pH 6.0, and 100 μL of crude enzyme was added to this substrate. The increase in absorbance was measured at 240 nm for 1–10 min at 25 °C. One unit of enzyme activity (U) was defined as the amount of enzyme which releases 1 μM of unsaturated uronide per min, based on molar extinction coefficient (5.55×103) of the unsaturated product (Barnby et al., 1990).

2.9. Enzyme extraction and purification

All extraction and purification steps were performed at temperature not exceeding 5 °C. The crude enzyme samples were partially purified by fractionation using ammonium sulphate (Merck) with